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Identification of Biomarkers in Amyotrophic Lateral Sclerosis

Mestrado em Biologia Molecular e Genética

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a progressive fatal neurodegenerative disease of the motor system. It is characterized by the death of the cortical, brainstem and spinal motor neurons.

Most people develop ALS between the ages of 40 and 70 and the diagnosis relies on clinical observation and neurophysiological studies. Major symptoms are weakness, fatigue and progressive paralysis of all muscles.

Between 90-95% of ALS cases are sporadic (sALS), without a genetic basis, and the remaining 5-10% cases are familial (fALS) and autosomal dominant inherited. Among these familial ALS mutations, about 20% occur in the gene for copper, zinc-superoxide dismutase (SOD1). When enzymatic activity of SOD1 is affected, the anion superoxide levels increase, generating cellular oxidative stress. In fact, the erythrocytes of patients with ALS present mutant SOD1 and dysregulation of the enzyme activity of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase.

At the moment three critical questions are still open: understanding the etiology of the disease, identification of molecular biomarkers and finding effective therapeutic agents.

Red Blood Cells (RBCs) transport oxygen to the tissues and carbon dioxide from the tissues. Erythrocytes also contribute to the regulation of vascular tone both by affecting blood viscosity and by capturing and releasing of vasoactive agents, such as nitric oxide (NO).

Nitric oxide (NO) is a signalling molecule produced in the central nervous system (CNS) that modulates neurological activity and blood flow. As a participant in oxidative stress, NO can mediate neuroprotection or neurotoxicity. As a neurotoxic factor, NO promotes oxidative stress through its reaction with superoxide anion to form peroxynitrite, and subsequently, other reactive derivatives. As a whole, these Reactive Nitric Oxide Species (RNOS), can induce DNA strand breaks, lipid peroxidation, protein nitration and nitrosylation, mitochondrial damage and cell apoptosis, contributing to neuroinflammation processes, thought to be involved in ALS. Once synthesized in endothelial cells by Endothelial Nitric Oxide Synthase (eNOS), NO can also diffuse to the adjacent smooth muscle cells or to the vascular lumen. When in the muscle, NO leads to muscle relaxation. When diffused to vascular lumen, NO is captured by RBCs that store and release this molecule, regulating erythrocyte aggregation and deformability and affecting their ability to transit across the capillaries. When scavenged by the erythrocyte, NO undergoes various reactions from which GSNO, nitrite and nitrate, relatively inert compounds, are produced.

Acetylcholinesterase (AChE) is located on the outer surface of the erythrocyte membrane, where it is known as a marker of membrane integrity, maintaining normal blood rheology and tissue oxygenation. Non-neuronal acetylcholine (ACh) may be regarded as a modulator of erythrocyte

hemorheological properties and as an essential cellular signalling molecule that contributes to the maintenance of cellular homeostasis, having a central role in the microvasculature. It exists in blood circulation and acts as an effector of vasodilation, since it stimulates the production of NO from eNOS, depending on the degree of endothelium integrity. ACh may also have an important role in the mobilization of NO and its derivatives in erythrocytes. In this mechanism, binding of circulating ACh with AChE in the erythrocyte membrane initiates a signal transduction mechanism that involves Gi and band 3 proteins, stimulating NO efflux and mobilization of nitrite and nitrate. As mentioned above, the RBCs of ALS patients present an excess of oxygen reactive species, which raises the hypothesis of their implication in erythrocyte biochemical and hemorheological functions.

The main objective of this research was to evaluate and compare the biochemical and hemorheological parameters in RBCs of patients with ALS and of healthy donors. Therefore, this project was expected to identify a parameter that once increased or diminished in the disease may serve as a biomarker. To achieve these goals, venous blood samples from 40 healthy donors and 82 ALS patients were collected after formal consent. Tests considering erythrocyte aggregation and deformability, NO efflux, AChE enzymatic activity and intraerythrocytic concentration of nitrite, nitrate and GSNO were performed.

Erythrocyte deformability and AChE activity were increased in patients with ALS in comparison to healthy donors. On the other hand, NO efflux and concentration of intraerythrocytic nitrite were lower in the disease. No significant differences were observed on the other parameters studied. However, considering ALS patients, there was a relation between the level of intraerythrocytic nitrite and AChE activity and between the efflux of NO and the Respiratory Function of patients. The results of the present study indicate that erythrocyte may contribute to control the NO bioavailability at microcirculatory level, presenting favourable deformability in the dysfunctional endothelium associated to the disease. Moreover, erythrocyte AChE should be further explored as a potential biomarker for ALS.

Keywords: Amyotrophic Lateral Sclerosis, Erythrocyte, Nitric oxide, Acetylcholinesterase, Nitrite

Resumo

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa fatal e de rápida progressão, caracterizada pela degeneração dos neurónios motores do córtex motor, tronco cerebral e medula espinhal.

A maioria dos doentes desenvolve ELA entre os 40 e 70 anos de idade e o diagnóstico baseia-se na observação clínica e testes neurofisiológicos. Os principais sintomas são fraqueza e paralisia progressiva de todos os músculos.

Entre 90-95% dos casos de ELA são esporádicos (não têm base genética) e os restantes 5-10% dos casos são familiares e de transmissão autossómica dominante. De entre as mutações características de ELA familiar, cerca de 20% ocorrem no gene que codifica para a enzima Cu/Zn superóxido dismutase 1 (SOD1). Uma vez alterada a actividade enzimática da SOD1, ocorre um aumento dos níveis de anião superóxido, o que contribui para o stresse oxidativo celular. De facto, os eritrócitos de doentes com ELA apresentam mutações na SOD1, às quais acresce desregulação da actividade enzimática do glutatião peroxidase, glutatião reductase e desidrogenase da glucose-6-fosfato.

A etiologia da doença é ainda desconhecida mas o stresse oxidativo, disfunções no transporte axonal e nas mitocôndrias, excitotoxicidade induzida por glutamato e neuroinflamação são alguns dos mecanismos apontados para explicar a degeneração dos neurónios motores.

Actualmente, falta ainda esclarecer três questões de máxima importância em respeito a esta incapacitante doença: compreender a sua etiologia, identificar biomarcadores moleculares que possam ajudar no diagnóstico e descobrir uma terapêutica efectiva.

Os glóbulos vermelhos transportam oxigénio dos pulmões para os tecidos e dióxido carbono destes para os pulmões, contribuindo também para a regulação da viscosidade sanguínea através da captação e libertação de agentes vasoactivos, como o monóxido de azoto (NO), e da modulação das propriedades mecânicas.

O NO é uma molécula sinalizadora produzida no sistema nervoso central que modula a actividade neurológica e o fluxo sanguíneo. Uma vez que participa no stresse oxidativo, o NO medeia mecanismos de neuroprotecção e neurotoxicidade. Como factor neurotóxico, promove o stresse oxidativo através da sua reacção com o anião superóxido para formar peroxinitrito e, consequentemente, outros metabolitos reactivos. Estas espécies reactivas de monóxido de azoto (RNOS) podem induzir a quebra de cadeias de DNA, peroxidação lipídica, nitração e nitrosilação de proteínas, disfunção mitocondrial e apoptose, contribuindo para os processos de neuroinflamação, descritos na ELA. Uma vez sintetizado pela enzima endotelial constitutiva, denominada sintase do

monóxido de azoto (eNOS), o NO pode difundir para as células musculares lisas adjacentes ou para o lúmen vascular. Uma vez no músculo liso, leva a relaxamento muscular. Quando difundido para o lúmen vascular, o NO é capturado pelos glóbulos vermelhos, que o armazenam e libertam, regulando assim a agregação e deformabilidade eritrocitárias, afectando a capacidade que estas células têm em circular pelos capilares.

O influxo de NO para o eritrócito faz-se inicialmente por ligação à proteína de membrana band-3 e depende do grau de fosforilação da mesma. Uma vez dentro do glóbulo vermelho, o NO conjuga-se com a hemoglobina, dependendo do seu grau de desnaturação, e com oxigénio, induzindo a produção de metahemoglobina. É da combinação do NO com as diferentes moléculas e proteínas existentes no eritrócito que resultam o S-nitrosoglutatião (GSNO), nitritos e nitratos, compostos relativamente inertes. O GSNO é produzido pela conjugação do glutatião com o NO dentro do eritrócito e funciona como reservatório, transportador, dador e mediador endógeno de NO, sendo considerado uma molécula antioxidante. Por sua vez, os nitritos e nitratos resultam essencialmente da decomposição dos peroxinitritos, mas também da ligação do NO a oxigénio e hemoglobina.

A acetilcolinesterase (AChE) está localizada na membrana externa dos eritrócitos, onde é conhecida como marcador da integridade de membrana dos mesmos, uma vez que ajuda a manter a normal circulação sanguínea e oxigenação dos tecidos. A acetilcolina (ACh) não-neuronal, substracto natural da AChE, poder ser considerada um modulador das propriedades hemoreológicas dos eritrócitos e uma molécula sinalizadora que contribui para a manutenção da homeostasia celular, tendo um papel central na microvasculatura. A ACh existe na circulação sanguínea e actua como efector da vasodilatação, uma vez que estimula a produção de NO pela eNOS, dependendo do grau de integridade da membrana. Desta forma, a ACh também participa na mobilização de NO e seus derivados no eritrócito. Para tal, a ligação da ACh plasmática à AChE da membrana do glóbulo vermelho inicia um mecanismo de transdução de sinal que envolve as proteínas Gi e band-3 de membrana, estimulando o efluxo de NO do eritrócito e a mobilização de nitritos e nitratos. Como mencionado anteriormente, os eritrócitos de doentes com ELA apresentam um excesso de espécies reactivas de oxigénio, o que sugere a implicação das mesmas em funções bioquímicas e hemoreológicas.

O objectivo principal deste estudo foi avaliar e comparar os parâmetros hemoreológicos e bioquímicos dos eritrócitos de doentes de ELA com os de dadores saudáveis. Para além disso, o projecto pretendeu identificar um parâmetro que, uma vez aumentado ou diminuído na doença, por comparação à situação fisiológica normal, se pudesse destacar como biomarcador.

Para tal, fez-se colheita de amostras sanguíneas de 40 controlos e de 82 doentes de ELA, depois de todos os participantes terem assinado o consentimento informado. Foram feitos testes que

estimassem a agregação e deformabilidade eritrocitárias, o efluxo de NO, a actividade enzimática da AChE e as concentrações intraeritrocitárias de nitritos, nitratos e GSNO.

Verificou-se que a deformabilidade e actividade da AChE estavam aumentadas nos doentes de ELA por comparação aos controlos. Por outro lado, o efluxo eritrocitário de NO e concentração intraeritrocitária de nitritos foram menores na doença. Não foram observadas diferenças significativas entre o grupo controlo e de doentes quanto à agregação e concentrações intraeritrocitárias de nitratos e GSNO. Porém, considerando exclusivamente os doentes de ELA, foi verificada uma relação entre a concentração de nitritos existente dentro dos eritrócitos e a actividade da AChE, na qual os nitritos, apesar de existirem sempre em baixas concentrações dentro dos glóbulos vermelhos dos doentes, estão aumentados em duas gamas de valores específicos de actividade da AChE. Uma vez que este aumento de nitritos é concordante com o efluxo de NO do eritrócito para os mesmos valores de actividade de AChE, pode ser sugerido um mecanismo compensatório no qual há menos libertação de NO quando os nitritos estão reduzidos no interior do glóbulo vermelho e mais efluxo de NO em valores de AChE para os quais os nitritos existem mais no interior do eritrócito. Foi igualmente demonstrada uma relação entre o efluxo de NO e a Função Respiratória dos participantes, na qual concentrações superiores de NO fora do eritrócito correspondem a uma pior função respiratória. Assim, é legítimo concluir que se o NO fosse mantido no interior do glóbulo vermelho, a Função Respiratória seria mais facilmente assegurada, ou seja, seria benéfico que o eritrócito actuasse como captador e reservatório de NO. Contudo, o estudo mostrou que o NO não é mantido no eritrócito, o que, mais uma vez e considerando o sistema pulmonar, pode ser justificado por um mecanismo compensatório no qual, quando a Função Respiratória está comprometida, o eritrócito liberta mais NO para que este possa ser utilizado pelos tecidos pulmonares.

Os resultados do presente estudo indicam que o eritrócito pode contribuir para a biodisponibilidade do NO, apresentando uma deformabilidade favorável num endotélio disfuncional, como o descrito na ELA. Adicionalmente, os resultados sugerem que a AChE do eritrócito possa vir a ser um marcador na ELA. Para tal propõe-se a continuação deste estudo com a introdução de mediadores pró e anti-inflamatórios e a análise da associação com os valores da AChE.

Palavras-chave: Esclerose Lateral Amiotrófica, Eritrócito, Monóxido de azoto, Acetilcolinesterase, Nitritos

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Abbreviations

Ach – Acetylcholine

AChE – Acetylcholinesterase

ALS – Amyotrophic Lateral Sclerosis

cGMP - Guanosine 3',5'-Cyclic Monophosphate

CSF - cerebrospinal fluid

EI - Elongation Index

eNOS - Endothelial Nitric Oxide Synthase

ER - Endoplasmic Reticulum

fALS – Familial Amyotrophic Lateral Sclerosis

FVC - Forced Vital Capacity

iNOS - inducible Nitric Oxide Synthase

GSH - Glutathione

GSNO – S-Nitrosoglutathione

Hb - Haemoglobin

metHb - Methemoglobin

MHC - Major Histocompatibility Complex

mtNOS - Mitochondrial Nitric Oxide Synthase

nNOS - Neuronal Nitric Oxide Synthase

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

PaO2 - Oxygen Partial Pressure

RBC – Red Blood Cell

RNOS - Reactive Nitric Oxide Species

ROS – Reactive Oxygen Species

sALS – Sporadic Amyotrophic Lateral Sclerosis

SOD1 - Copper, Zinc-Superoxide Dismutase

SPSS – Statistical Package for the Social Sciences

VSMC – Vascular Smooth Muscle Cell

1. Introduction

1.1 - Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a fatal and rapidly progressive neurodegenerative disease of the motor system (1). It is one of the most common adult onset motor neuron diseases and is characterized by the death of the cortical, brainstem and spinal motor neurons (2). The degeneration of these neurons in the upper and lower motor neuron systems has been demonstrated to be mediated by apoptotic processes (3).

Decomposing the name and citing from the ALS Association: "'A' means no. 'Myo' refers to muscle, and 'Trophic' means nourishment. (…) When, a muscle has no nourishment it 'atrophies' or wastes away." 'Lateral' identifies the spinal cord area where the corticospinal tract is located. "As this area degenerates it leads to scarring or hardening ('sclerosis') (…). When the motor neurons die, the ability of the brain to initiate and control muscle movement is lost." (4).

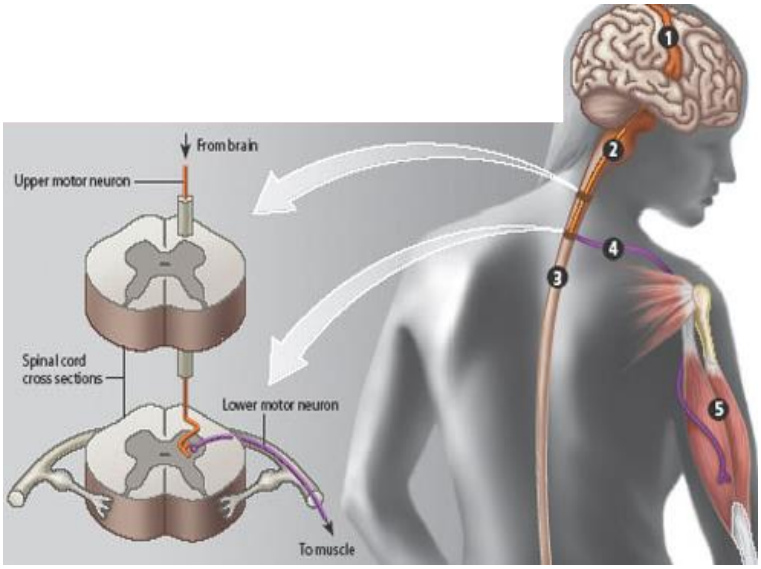

Figure 1 - Diagram illustrating anatomic targets of ALS. From: http://www.imagekb.com/amyotrophic-lateral-sclerosis

Although ALS can have a heterogeneous clinical presentation, patients develop weakness, fatigue and progressive paralysis of all the skeletal, bulbar and respiratory muscles, which originate respiratory insufficiency (5) and, consequently, results in a high risk of death within 2-5 years after symptom onset (4).

Most people developing ALS are between the ages of 40 and 70, with an average age of 55 at the time of diagnosis. ALS is 20% more common in men than in women but with increasing age, the incidence of ALS becomes similar in the two genders (4).

The incidence of ALS is 1-2 individuals per 100,000 while the prevalence is of 6-8 per 100,000 of the total population (6).

Nowadays, ALS is considered a multi-factorial and multi-systemic disorder in which modifications of the crosstalk between neuronal and non-neuronal cell types might influence the progression of the disease (6).

The disease was firstly well described in 1869 by the French neurologist Jean-Martin Charcot (4).

1.1.1) Genetics

Between 90-95% of ALS cases are sporadic (sALS), without a genetic basis, and the remaining 5-10% cases are familial (fALS) and predominantly autosomal dominant inherited (1). More than twenty molecular mutations have been discovered in familial ALS and most of them cause mutated protein accumulation, aggregation and result in endoplasmic reticulum (ER) stress (7). However, about 20% of these familial ALS mutations are associated with modifications in the gene for copper, zinc-superoxide dismutase (SOD1) that lead to a gain of toxic function of this enzyme (2). A large number of familial cases derives from C9Orf72 intronic expansion.

SOD1 is an abundant copper- and zinc-containing intracellular enzyme (2) with a free radical scavenging function (8), since it catalyses the dismutation of superoxide anion to hydrogen peroxide and oxygen in a two-step redox reaction (1; 5). For this reason, point mutations and a small deletion in the Cu/Zn superoxide dismutase gene on chromosome 21 supports the free radical hypothesis for the pathogenesis of ALS (9).

Although, SOD1 mutations are specially associated with fALS cases, they can also occur in sporadic cases and cause protein misfolding that increases the exposure of the active site copper.

Individual genetic susceptibility and epigenetic mechanisms are likely to influence disease onset, progression and phenotypes.

The similar clinical presentation in sporadic and familial ALS suggests that the two forms of the disease share common pathogenic mechanisms (5).

1.1.2) Pathogenesis

Although the pathogenesis of ALS is still unknown, some mechanisms are hypothesized to explain motor neuron degeneration. Some examples are:

- Oxidative stress: It is the result of unregulated production of reactive oxygen species, such as hydrogen peroxide, peroxynitrite, superoxide anion and hydroxyl radicals. Neuronal tissues from patients with sALS show oxidative damage to proteins, lipids, and DNA, which results in motor neuron degeneration and astrocyte dysfunction (1; 5).

The discovery of mutations in the anti-oxidant enzyme SOD1, causative of ALS, has supported the relevance of oxidative stress in the disease (1).

- Glutamate induced excitotoxicity: There is evidence of increased levels of glutamate in the cerebrospinal fluid and plasma of patients with ALS (10). This occurs due to decreased glutamate transport in the brain and spinal cord and an increased expression of glutamate receptors permeable to excessive influx of Na^+ and Ca^{2+} ions on motor neurons in ALS (11). *Bondy* and *Lebel* showed that glutamate excitotoxicity may result in the production of free radicals and thereby cause cell death (12).
- Protein misfolding and aggregation: Gene mutations found in fALS generate mutated protein accumulation, aggregation, or both in the cytoplasm of motor neurons and glial cells (13). This is likely to result in ER stress, leading to more misfolded proteins, which are also considered a potential cause of sALS (14). Nevertheless, mutant SOD1 is the most widely protein studied that tends to misfold and form aggregates and inclusions in muscle cells and motor neurons (6).
- Mitochondrial dysfunction: It is largely associated with oxidative damage and, consequently, neurodegeneration, by the production of inadequate levels of ATP and reactive oxygen species (like superoxide anion), increment of calcium-mediated excitotoxicity, or by triggering apoptosis (1; 15).
- Neuroinflammation: It is marked up by regulated expression of major histocompatibility complex (MHC) molecules, elevations in proinflammatory cytokines and chemokines, leukocyte, proapoptotic factors and reactive oxygen intermediates within the neuronal tissues (16; 15).
- Genetic mutations: Most of the mutations that occur in these diseases are point mutations, responsible for destabilizing protein conformation. The resulting misfolded proteins may then aberrantly accumulate and affect several cellular functions (axonal transport alterations, mitochondrial and/or proteasome dysfunctions) (17; 18).
- S-nitrosylation: S-nitrosylation is the process by which Nitric Oxide (NO) modify critical cysteine residues on proteins leading to the protein acquirement of NO groups from a pool of intracellular S-nitrosylated peptides.

  Aberrant depletion of intracellular Nitric Oxide Synthases (NOSs) contributes to motor neuron death in ALS, and raises the possibility that deficient S-nitrosylation is a general mechanism of disease pathogenesis. SOD1 mutants are an example of the increased denitrosylase activity (particularly prominent in mitochondria), which contributes to motor neuron death in ALS,

through the depletion of intracellular NOSs. However, either excessive or deficient levels of protein S-nitrosylation may contribute to the disease (2).

- Protein nitration: It is an oxidative postranslational modification limited to specific tyrosine residues on proteins that leads to significant changes in protein structure and function (altered enzyme activity, increased propensity to form aggregates and ability to stimulate immunogenic response) that may contribute to altered cell and tissue homeostasis. Excess levels of reactive oxygen species in the presence of •NO or •NO-derived metabolites lead to the formation of nitrating species such as peroxynitrite (15; 19).
- Cytoskeletal abnormalities
- Proteasome inhibition and autophagy
- Transcriptional dysfunction (neuronal tissue from patients with sALS shows oxidative damage to proteins, lipids, and DNA) (5).

Although the sequence of pathogenic events is not clearly established, most of them are intimately correlated (genetic mutations lead to protein misfolding and consequent aggregation, which causes impaired axonal transport, mitochondria damage and apoptosis that may also contribute to production of free radical species that result in oxidative stress, glutamate induced excitotoxicity and neuro-inflammation).

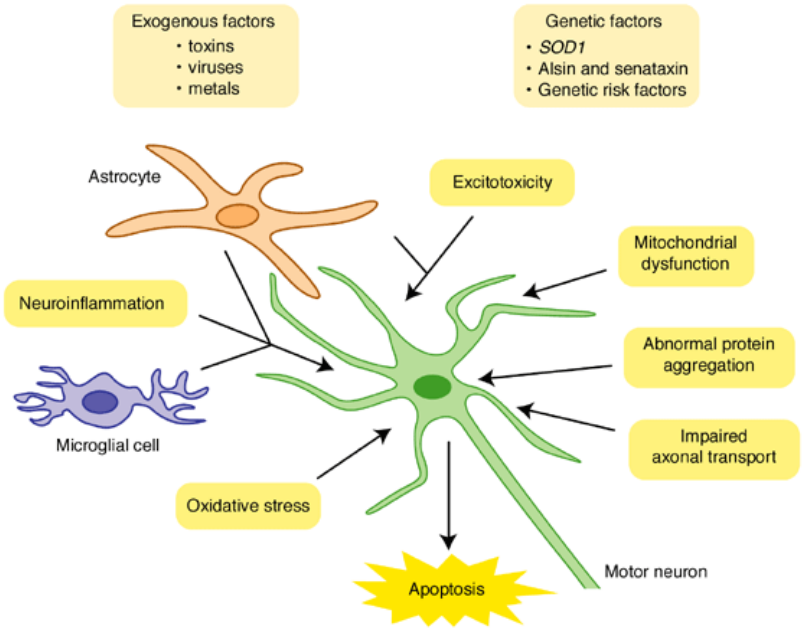

Figure 2 - Overview diagram summarising some of the theories of ALS pathogenesis. From: Expert Reviews in Molecular Medicine (2006) Cambridge University Press. Available in http://journals.cambridge.org/fulltext_content/ERM/ERM8_11/S1462399406010854sup018.htm

1.1.3) Risk factors

The pathological mechanisms presented above, particularly the generation of oxidative stress, can be triggered by some risk factors. Beyond them are agricultural chemicals, heavy metals, military service, excessive physical exertion, chronic head trauma and diet, with a modest risk association with ALS, and smoking with a stronger association (5). Age, race and gender might also be considered (4).

1.1.4) Diagnosis

ALS is difficult to diagnose. Once there is no biomarker, the diagnosis of the disease is essentially clinical and supported by electromyography (1). Other investigations are relevant to exclude other clinical conditions, such as neuroimaging studies (magnetic resonance imaging), lumbar puncture and muscle and/or nerve biopsy (4).

Laboratory tests might also be useful since they reveal changes in oxidative stress biomarkers (such as pro-inflammatory cytokines and nitrated proteins), which are elevated in cerebrospinal fluid (CSF), plasma, and urine of patients, suggesting that abnormal oxidative stress is equally generated outside of the central nervous system (5). Blood and urine studies may, for instance, include urine collection for heavy metals (4).

1.1.5) Clinical manifestations

The symptoms of ALS and the rate at which they progress can vary widely among the patients (4). Some common clinical manifestations are progressive muscle weakness, fasciculation (spontaneous and intermittent activation of the muscle fibres), paralysis, tripping, muscle cramps, twitching, abnormal fatigue of the limbs, slurred speech, difficulty with chewing and swallowing, malnutrition and cachexia, cognitive disorders (learning, memory, perception and problem solving may be affected), depression and anxiety disorders, uncontrollable periods of laughing or crying and particular respiratory muscle weakness that leads to respiratory failure - the cause of death (despite the permanent ventilatory support in the late stages of the disease) (4; 5; 11; 16; 20).

Some of the previous symptoms (malnutrition, respiratory failure, and psychological stress) may lead to the accumulation of oxidative stress (5).

It is important to note that since ALS attacks only motor neurons, the senses of sight, touch, hearing, taste and smell are not affected (4).

1.2 - Erythrocytes

Neurons are the main affected cells in this “Motor Neuron Disease”, as previously described. However, erythrocytes assume functions in the body that might explain the basis of some pathogenic mechanisms of ALS, reason why its study is of extreme importance.

Red Blood Cells (RBCs) are the major cell component of blood. Their principal function is the transport of oxygen to the tissues and carbon dioxide from the tissues (21). RBCs also contribute to the regulation of vascular tone both by affecting mechanical properties (aggregation and deformability) and capturing and releasing of vasoactive agents, such as nitric oxide (NO) (22).

These functions of RBC might assume preponderant roles in disease since, as a consequence of being a vital biological sensor, it has the ability to reverse or reduce pathological situations (for instance, capturing NO in highly oxygenated tissues and releasing NO in hypoxic tissues) (23; 24).

The capture and donation of NO and oxygen is associated with the state of relaxation and tension of haemoglobin (the major protein of erythrocytes), respectively (25).

1.3 - Biomarkers in erythrocytes

1.3.1) Aggregation and deformability

Protein hyper aggregation is associated with inflammatory processes and pathological situations. However, erythrocytes may also form aggregates, particularly when blood flow is destabilized and low shear stress dominates (23).

Such aggregation can be caused not only by the presence of a variety of macromolecules (especially fibrinogen) in plasma, but also by surface properties of RBCs (for instance, depending on the level of phosphorylation/dephosphorylation of the surface protein band 3) (22; 26).

Erythrocyte aggregation adversely affects blood flow dynamics in the microcirculation, reason why it normally occurs in postcapillary venules, leading to an increase in blood viscosity and flow pre-stasis and to a decrease in the efficiency of oxygen transport (27; 28).

The level of aggregation can rise enormously in association with a wide variety of clinical conditions with inflammatory response (26), which justifies its study in ALS.

Erythrocyte aggregation is intimately associated with deformability, another hemorheological parameter.

Deformability is the erythrocyte ability to reversibly adopt a new configuration when subjected to applied mechanical forces. It can be measured in Elongation Index (EI), in percentage, and an increased EI indicates greater RBC deformability (29).

The ability of the entire RBC to deform is of crucial importance for the maintenance of normal circulation, since it allows the passage of erythrocytes through narrow capillaries and reduces blood viscosity (30; 31).

The major determinants of RBC deformability are cell geometry, cytoplasmic viscosity and membrane mechanical properties and composition (29). In fact, the configuration of membrane proteins (like band-3, spectrin, actin and protein 4.1) can be modulated by phosphorylation or dephosphorylation states, with a decrease or increase in the interactions between erythrocyte membrane and cytoskeleton, leading to higher or lower erythrocyte deformability values, respectively (32).

Overall, a significant modification in the erythrocyte biorheology has physiological relevance, once it encompasses changes in blood viscosity, affects erythrocytes ability to transit across the capillaries, modulates the distribution of blood in the several vascular territories and therefore tissue oxygenation (33).

1.3.2) Nitric Oxide (NO)

Nitric oxide (NO) is a gas signalling molecule produced in the central nervous system (CNS) and in other extra neuronal systems that can diffuse across lipid membranes or be transported through cells and tissues to act far from its source. It modulates diverse biological functions, especially neurological activity and blood flow (15; 16; 34).

NO is produced by a group of enzymes denominated Nitric Oxide Synthases (NOSs). There are four members in the NOS family: neuronal NOS (nNOS), which is constitutively expressed in CNS neurons, and is activated by calcium; endothelial NOS (eNOS), which is constitutively expressed in endothelial cells and some astrocytes, and is regulated by calcium and phosphorylation; inducible NOS (iNOS), which is not normally expressed but is induced in glia and endothelial cells by hypoxia, pro-inflammatory cytokines and pathogens; and mitochondrial NOS (mtNOS), the existence and origin of which are controversial (35; 36).

All isoforms of NOS catalyse the oxidation of L-arginine in L-citrulline and NO, in a two-step reaction requiring NADPH and oxygen, where N-hydroxyl-L-arginine is the intermediate metabolite (37).

This gas molecule mediates diverse physiological or pathological functions. As a participant in oxidative stress, and depending on environmental conditions (for example, interaction between antioxidant defences and cellular repair mechanisms), NO can mediate neuroprotection (acting as a protective antioxidant) or neurotoxicity (intensifying oxidative stress) (16).

Therefore, as a protector and as a signal transduction agent, in the brain and CNS, it participates in modulation of neurotransmission, memory and synaptic plasticity (15) and inhibits apoptosis (16), what makes it a valuable therapeutic agent in ageing-associated diseases like ALS. In addition, NO is also important in various organ systems, acting as a modulator of endothelial function (controlling the balance between vasoconstriction and vasodilation, preventing platelet aggregation (38) and inhibiting the proliferation of vascular smooth muscle cells (VSMCs) (35)), contributing to muscle relaxation (39), killing intracellular pathogens (34), scavenging radicals and limiting lipid peroxidation (16).

On the other hand, NO can change from a physiological neuromodulator to a neurotoxic factor and become injurious under pathological conditions, where it is produced in an excessive amount. In these situations, NO promote oxidative stress through its reaction with superoxide anion to form peroxynitrite, and subsequently, other reactive derivatives (15). As a whole, these Reactive Nitric Oxide Species (RNOS), can induce DNA strand breaks, lipid peroxidation, and protein nitration (40) and nitrosylation, mitochondrial damage and cell apoptosis (36).

These contrasting roles are illustrated in ALS, where the same concentrations of NO that in some models promote the survival of motor neurons, under normal conditions, can also promote tissue damage and neurodegeneration (15).

Nevertheless, although NO is produced in CNS as referred above, once synthesized in endothelial cells, NO can also diffuse to the adjacent smooth muscle cells or to the vascular lumen (41). When in the muscle, guanosine 3',5'-cyclic monophosphate (cGMP) is activated by NO, what leads to subsequent muscle relaxation (39). When, diffused to vascular lumen, NO is captured by RBCs via binding to haemoglobin, inducing the

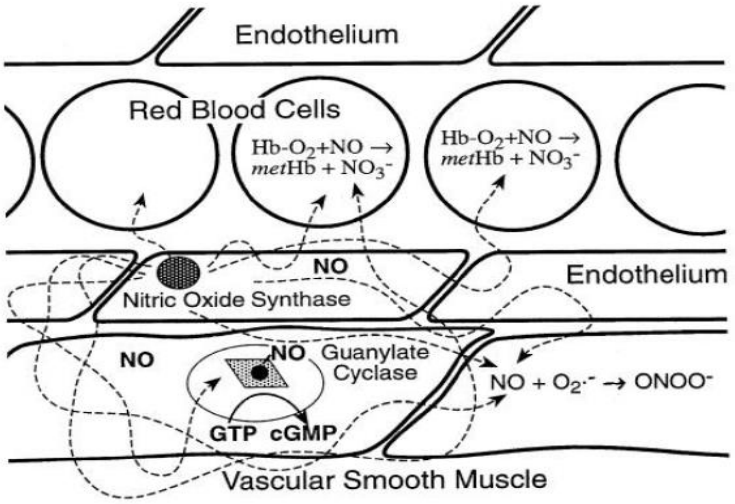

Figure 3 - Principal reactions of nitric oxide in tissues and red blood cells. Abbreviations: Hb, haemoglobin; metHb, methemoglobin; NO, nitric oxide; NO-3, nitrate; GTP, guanosine triphosphate; cGMP, guanosine 3',5'- cyclic monophosphate; O2-, superoxide anion; ONOO-, peroxynitrite. From: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly (1996).

formation of methemoglobin (metHb) (29). The influx of NO to RBCs occurs through the protein of membrane band 3 and depends on the degree of phosphorylation of this protein (42) and on the degree of denaturation of haemoglobin (43), what suggests that NO may have a role in regulating erythrocyte biorheological behaviour (aggregation and deformability) (29).

However, human RBCs also have the ability to synthesize their own NO, as demonstrated by the presence of the active eNOS isoforms in them (44). The mobilization of erythrocytic NO is dependent on its concentration, on circulating acetylcholine and on the cellular redox thiol status (35; 45).

Therefore, knowing that NO and RNOS are involved in oxidative stress and neuroinflammation processes, thought to contribute to ALS, and at the same time that NO is stored, produced and released by the erythrocytes, its study is justified in these cells.

1.3.3) Nitrite and nitrate

When scavenged by the erythrocyte, NO undergoes various reactions from which nitrite and nitrate, relatively inert compounds, are produced.

If oxidation of haemoglobin occurs, the superoxide anion is formed. Then, NO reacts with superoxide anion and produces peroxynitrite that decomposes into nitrite and nitrate (46).

NO and nitrite may also react directly with haemoglobin (Hb) to form nitrate and methemoglobin (47). On the contrary, nitrate could be converted to nitrite by the action of metHb reductase (48).

RBCs scavenge nitrite from the bloodstream when their concentration increases intensely in response to the release of NO from the erythrocyte in pathological situations (47).

1.3.4) S-nitrosoglutathione (GSNO)

S-nitrosoglutathione (GSNO) is a low molecular weight molecule, classified as an s-nitrosothiol (49). Glutathione (GSH), the most abundant cellular thiol, participates in important functions in the organism (detoxification of xenobiotics, cell homeostasis, radioprotection, and antioxidant protection (50; 51)) and exists in high concentrations inside erythrocytes. Here, their thiol group reacts with NO, forming GSNO, in an oxygen dependent process (52). GSNO can also be produced through oxidation of glutathione by peroxynitrite (53).

GSNO serves as a reservoir, transporter, donor and endogenous mediator of NO, being an antioxidant molecule (2). Moreover, with the production of GSNO, the formation of peroxynitrite is prevented by substrate competition (54), which along with the fact that this S-nitrosothiol also plays a fundamental role in vascular regulation homeostasis, illustrates its interest as a potential therapeutic agent (49).

Some evidence shows that GSNO is increased under inflammation conditions, where NO is increased (55). On the other hand, it was demonstrated that cells expressing the SOD1 mutants (as it may happen in ALS) had significantly lower GSNO levels as compared with cells expressing wild type SOD1 (2).

1.3.5) Acetylcholine (ACh) and Acetylcholinesterase (AChE)

Acetylcholine (ACh) is an important neurotransmitter. It is synthesized at nerve cells from choline and acetyl coenzyme A by a process mediated by choline acetyltransferase and is stored in presynaptic neurons. Upon the arrival at a nerve impulse, ACh is released and, once at the postsynaptic membrane, it initiates a cascade of actions that is terminated by acetylcholinesterase (AChE), which hydrolyses ACh to choline and acetic acid (56).

However, ACh and AChE are not restricted to the neuronal cholinergic system (57). It has been demonstrated that ACh exists in blood circulation and that it is produced by endothelial cells and T-lymphocytes. AChE can be located on the outer surface of the erythrocyte membrane, where it is known as a marker of membrane integrity (58), maintaining normal blood rheology and tissue oxygenation (59).

Non-neuronal ACh may be regarded as an essential signalling molecule that contributes to the maintenance of cellular homeostasis (57), having a central role in the microvasculature (60). It acts as an effector of vasodilation, since it stimulates the production of NO from eNOS in endothelial cells (61), depending on the degree of endothelium integrity (27).

ACh may also have an important role on the mobilization of NO and its derivatives in erythrocytes. In this mechanism, binding of circulating ACh with AChE of RBC membrane originates a signal transduction mechanism that involves the phosphorylation/dephosphorylation of band 3 protein by a G protein, what stimulates nitric oxide efflux and mobilization of nitrite and nitrate (46). These vascular events may occur under pathology, such as hypoxic and inflammatory conditions.

Furthermore, by stimulation of ACh and due to phosphorylation and consequent reduction of membrane-cytoskeleton interaction, erythrocyte deformability increases and erythrocyte aggregation

decreases in blood samples of healthy donors as it was demonstrated by *Mesquita et al.* (62), whereby ACh is a modulator of erythrocyte hemorheological properties (63).

Briefly, AChE may have a role in the signal transduction mechanism in response to ACh, which results in NO, nitrite and nitrate mobilization, in human erythrocyte suspensions (27), what routs the ACh–AChE active complex for therapeutic control of NO bioavailability in the RBC (61). The hypothesis previously raised and illustrated in Fig 4, has been proved by A. Carvalho *et al*. (46)

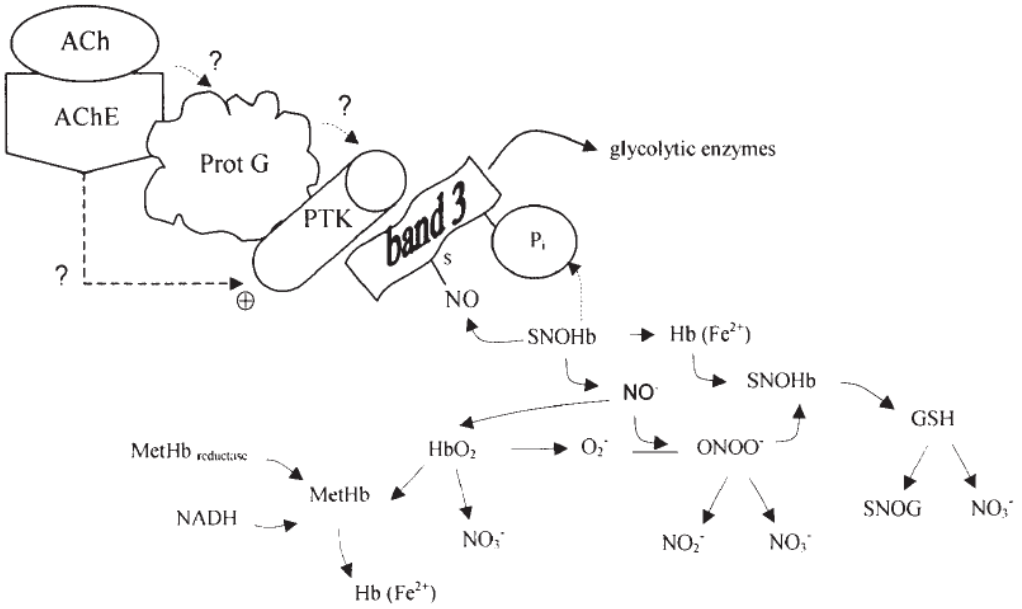

Figure 4 - Hypothesis for the possible AChE role in the signal transduction mechanism in response to the action of ACh on nitrite and nitrate production in human erythrocyte suspensions. Abbreviations: Pi, phosphate; NADH, nicotinamide adenine dinucleotide reduced; Hb, haemoglobin; NO, nitric oxide; O_2^-, superoxide anion; $ONOO^-$, peroxyinitrite; NO_3^-, nitrate; NO_2^-, nitrite; GSH, glutatione reductase; SNOG, S-nitrosothiol; HbO_2, oxyhaemoglobin; MetHb, methaemoglobin. From: Acetylcholine and Choline Effects on Erythrocyte Nitrite and Nitrate Levels (2004).

2. Objectives

As described herein, there is an association between ALS and the elevated concentration of NO derivatives, such as plasma nitrate, and the degree of oxygen reactive species, due to impairment of antioxidant enzymes activity. Moreover, other parameters characteristic of inflammation, which is augmented in this pathological situation, may also influence the erythrocytes' function.

Therefore, the main purpose of this work was to evaluate and compare the biochemical and hemorheological parameters in RBCs of patients with ALS and of healthy donors.

Briefly, this project was expected to identify a parameter or a combination of parameters that, once increased or diminished in the disease, in contrast to the physiologic normal situation, may serve as biomarkers. This is of extreme interest to facilitate the process of diagnosis of ALS and of discovery of future therapeutics.

3. Materials and methods

3.1 - Groups of study

3.1.1) Characterization of groups of study

This study was performed in two different groups of participants: healthy donors, used as the control group, and patients with ALS.

In total, 40 healthy donors (20 women and 20 men) and 82 ALS patients (36 women and 46 men) were recruited.

3.1.2) Blood collection protocol

The control group used to establish the reference values for the studied parameters was achieved by the samples provided by healthy volunteers, under a partnership between Instituto Português do Sangue and the Biochemistry Unit of Instituto de Medicina Molecular.

Blood samples from patients with ALS were collected after a medical consultation with Professor Doctor Mamede de Carvalho, in Hospital de Santa Maria, and after the patients knew the purpose of the study and had signed an informed consent.

Every sample of human venous blood was collected from the forearm vein to a tube with the anticoagulant heparin.

The hemorheological and biochemical analysis were performed immediately upon arrival of samples to the laboratory, so that no property of the collected blood was lost with the passage of time and thus any measured parameter was compromised.

3.2 - Experimental procedure

3.2.1) Aggregation

Erythrocyte aggregation was determined by using the MA1 aggregometer from Myrenne (Roethen, Germany). This equipment consists of a rotating cone-plate chamber, which disperses the sample through high shear stress, and a photometer that determines the extent of erythrocyte aggregation. This parameter was assessed using 20 μL of blood during five seconds in stasis, first, and ten seconds, next, after dispersion of the blood sample (64).

3.2.2) Deformability

Erythrocyte deformability is expressed as the elongation index (EI), in percentage, and was determined for different values of shear stress (0.30, 0.60, 1.20, 3.00, 12.00, 30.00 e 60.00 Pa) using the Rheodyn SSD laser difractometer from Myrenne (Roetgen, Germany). This equipment determines deformability by simulating the shear stresses exerted by the blood flow and the vascular wall on the erythrocytes. For that purpose, 30 µl of blood were suspended in 2 ml of Dextran (a viscous medium) and injected into the device, in order to be placed between a rotating optical disk and a stationary disk, responsible for subject the erythrocytes to precise shear stresses which force them to deform to ellipsoids. The degree of deformability is evaluated by a laser beam which passes across the suspension and presented on a computer. The higher the elongation index, the higher the degree of deformability (65).

3.2.3) Nitric oxide (NO)

All parameters were performed in the laboratory in the same order in which they are described herein, whereby it is important to note that from the measurement of NO, included, the sample used was the suspension of erythrocytes, since the whole blood was centrifuged and the supernatant discarded.

For amperometric NO quantification, the *amiNO-IV* sensor (Innovative Instruments Inc. FL, USA) was used. The NO sensor was vertically immersed in erythrocyte suspension (1,5 µl of erythrocytes were diluted in 2,97 ml of NaCl 0,9%, pH 7,0) and the solution was homogenized by gently inversion. After a few minutes of stabilization of the sensor, 30 µL of acetylcholine (ACh 10 mM) were added to the erythrocyte suspension. As a consequence, the NO diffuses through the gas permeable membrane of the sensor and is then oxidized at the working platinum electrode, resulting in an exchange of electrons between NO and the electrode and, subsequently, in electric current. This alteration is proportional to the amount of NO released from erythrocytes after Ach stimulation and is continuously monitorized with an inNO-TM software (version 1.9 supplied by Innovative Instruments Inc.) installed on a computer (66).

The concentration of NO is obtained using the following formula:

[NO] (nM) = (ΔpA – 41,622) / 60,987, where ΔpA = Max – Min

3.2.4) Acetylcholinesterase (AChE)

At the beginning of the procedure, eppendorfs with acetylthiocholine, quinidine sulfate, eserine sulfate and dithionitrobenzoic acid (DTNB) / Ellman's reagent were removed from the freezer and placed in a bath at 25 ˚C.

Then, 20 µl of the centrifuged blood were diluted in 10 ml of 0,1M Phosphate (Pi) buffer pH 8,0. This mixture was vortexed and, then, 9 ml were equally distributed among the three test tubes, corresponding to a blank (B) and two samples (S1 and S2). 100 µl of eserine sulfate were added to the blank tube (after removed from the bath and agitated in vortex).

A fourth tube served as support for the Reaction Mixture comprising 1000 µl of DTNB, 800 µl of acetylthiocholine and 400 µl of quinidine sulfate (after removed from the bath and stirred in the vortex). After mixing, 50 µl of the Reaction Mixture were added to each test tube: B, S1 and S2. All tubes were vortexed and placed in the bath for 5 minutes. At this time, 100 µl of eserine sulfate were added to the two sample tubes (S1 and S2).

The amount of enzyme activity of AChE is obtained from reading the absorbance of the solutions of test tubes at 412 nm, in Spectronic 20 Genesys, and according to the formula (67):

$$\text{AchE (U/min/mg Hb)} = \frac{\left[\frac{\text{Abs Sample 1 (nm)} + \text{Abs Sample 2 (nm)}}{2}\right] - \text{Abs Blank (nm)}}{(\text{Haemoglobin of diluted blood (g/dL)} / 2)} \text{ x } 3000$$

3.2.4.1) Haemoglobin of total and diluted blood

The value of haemoglobin (g/dL) of whole blood was read in Poch-100iV device - Hematology Analyzer with an eppendorf with 100 µl of whole blood.

In order to obtain the haemoglobin value of diluted blood, 200 µl of the blood centrifuged previously were diluted in 800 µl of 0,1M Phosphate (Pi) buffer pH 0,8. The mixture was vortexed and 100 µl were transferred to an eppendorf for measuring the amount of haemoglobin in the same equipment. This haemoglobin value is used to calculate the enzyme activity of AChE.

3.2.5) Preparation of samples for the study of nitrite (NO^-_2), nitrate (NO^-_3) and S-nitrosoglutathione (GSNO)

For the study of the following three parameters, 400 µl of the centrifuged blood were subjected to haemolysis, by the addiction of 400 µl of deionized water, and haemoglobin precipitation,

by the addiction of 200 µl of 95% cold ethanol and 120 µl of cold chloroform. After vortex agitation, the mixture was centrifuged for 10 minutes at 3000 rpm. At the end, the supernatant was separated from the pellet (packed erythrocytes) to be used in the following steps.

3.2.6) Determination of the concentration of nitrite (NO^-_2) by Griess method

Nitrite concentration was measured with the spectrophotometric Griess reaction. Thus, two cuvettes were prepared: a blank and another with sample. In the blank cuvette, 33 µl of *Griess* reagent (previously prepared from a mixture of sulfanilic acid - component B - and NEDD – component A) were added to 966 µl of deionized water. In the sample cuvette, 33 µl of Griess reagent were added to 866 µl of deionized water and 100 µl of supernatant.

After a 30 minute incubation, the absorbance was read at 548 nm in Spectronic 20 Genesys. The formula used to calculate the concentration of NO^-_2 is (45):

$[NO^-_2]$ (µM) = (Sample Abs – 0,0008) / 0,002

3.2.7) Determination of the concentration of nitrate (NO^-_3) by Griess method

For nitrate measurement, this compound was first reduced to nitrite in presence of nitrate reductase.

To achieve this, a sample cuvette was prepared with 100 µl of supernatant, 25 µl of nitrate reductase and 25 µl of NADPH. After stirring, the cuvette was incubated for 30 minutes.

At the end of this period, another cuvette was prepared (blank for the spectrophotometric reading), with 50 µl of Griess reagent and 1450 µl of deionized water. At the same time, 50 µl of Griess reagent and 1300 µl of deionized water were also added to the sample cuvette.

Both cuvettes were incubated at room temperature for 30 minutes. Finally, the absorbance was read at 548 nm in Spectronic 20 Genesys and $[NO^-_3]$ calculated according to the following formula (45):

$[NO^-_3]$ (µM) = (Sample Abs – 0,0008) / 0,002

3.2.8) Determination of the concentration of S-nitrosoglutathione (GSNO)

A Reaction Mixture of component A (NEED) and B (sulfanilic acid) of Griess Reagent and Phosphate-Buffered Solution pH 7,4 (PBS) was prepared and agitated in the vortex.

Then, four cuvettes, two as blanks and two of samples (B1, B2, S1 and S2) were set. Into the cuvette B1, 33 µl of Reaction Mixture and 967 µl of deionized water were added. Cuvette B2 was pipetted up with 33 µl of Reaction Mixture, 10 µl of mercuric chloride ($HgCl_2$ 10 nM) and 957 µl of deionized water. For cuvette S1, 33 µl of Reaction Mixture, 100 µl of supernatant, 10 µl of $HgCl_2$ and 856 µl of deionized water were added. Finally, 33 µl of Reaction Mixture, 100 µl of supernatant, and 866 µl of deionized water were added in cuvette S2.

After gentle shaking, the four cuvettes were incubated for 20 minutes and, at the end of that time, the absorbance was read at 496 nm in Spectronic 20 Genesys. The calculation of [GSNO] was based on the following formula (68):

[GSNO] (µM) = (1084,5 x Δ Abs) + 5,99 , where Δ Abs = (Abs S1 – Abs S2) – Abs B2

3.3 - Statistical analysis

The statistical method chosen was ANOVA. Data was expressed in mean, Standard Deviation (SD) and the values obtained were considered statistically significant when P<0, 05.

Data was also presented in inter-quartiles IQ (Q25%-Q75%).

For statistical analysis SPSS (version 22.0) was the software employed and EXCEL was the informatics program used for producing and analysing the graphs.

4. Results

The results presented bellow make a comparison between the control group (40 participants) and patients with ALS (82 participants) considering all the parameters studied (Annex 1 presents an excerpt of the table that comprises all the results obtained in this project).

4.1 - Erythrocyte Aggregation

Figure 5 illustrates the erythrocyte aggregation among the two groups of study: the control group (CTR) and patients with ALS (ALS), in periods of analysis of 5 seconds and 10 seconds.

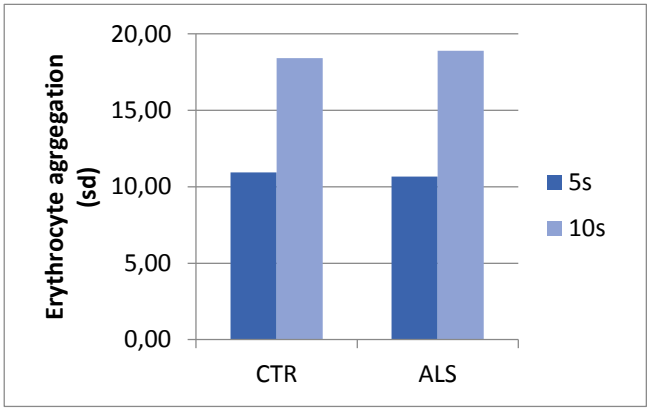

Figure 5 - Comparison of erythrocyte aggregation between controls and patients with ALS.

It is possible to conclude from the figure that no significant difference is observed in erythrocyte aggregation between healthy donors (5s: 10,94±2,26 sd; 10s: 18,41±4,64 sd) and patients (5s: 10,67±2,34 sd; 10s: 18,90±4,06 sd).

4.2 - Erythrocyte Deformability

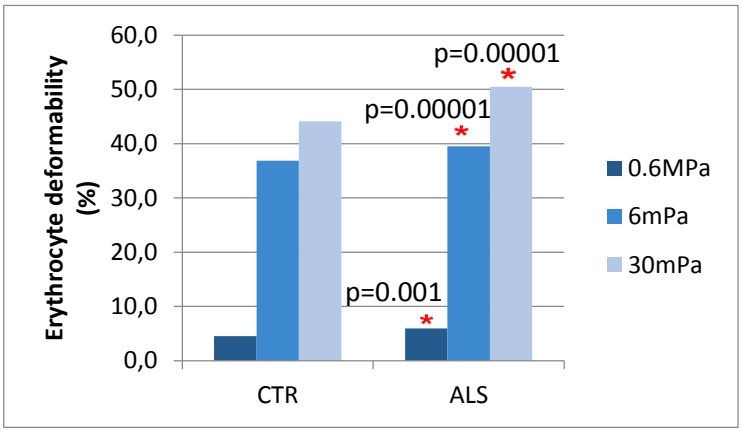

Figure 6 - Comparison of erythrocyte deformability between the two groups.

Figure 6 shows the RBCs deformability, expressed as the Elongation Index (in percentage), at the different values of shear stress at which they were subjected.

Observing the graph, it is possible to notice a statistically significant difference between the control group and patients with the disease. In fact, erythrocytes of patients with ALS have more deformability (0,6 mPa: 5,9±2,3%; 6 mPa: 39,5±4,0%; 30 mPa: 50,5±6,0%; $P < 0,05$) in comparison to healthy donors (0,6 mPa: 4,5±1,8%; 6 mPa: 36,8±2,9%; 30 mPa: 44,1±7,6%), for the three values of shear stress analysed.

4.3 - NO efflux

Figure 7 presents the difference of NO concentrations (in nM), regarding its efflux from the erythrocyte, between the control group and patients.

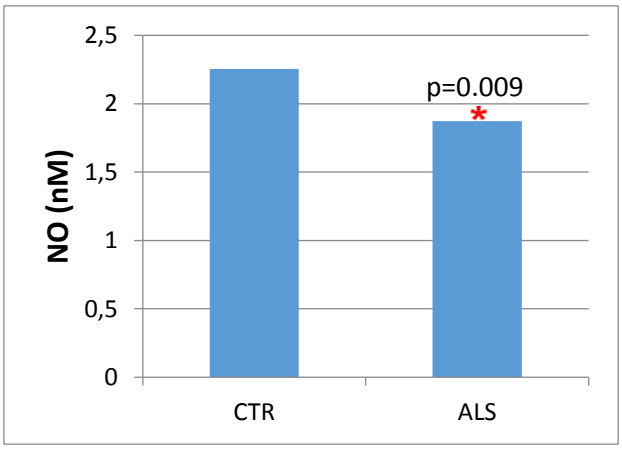

Figure 7 - Comparison of NO efflux between the control and disease group.

The concentration of NO outside the RBCs of patients with ALS (1,9±0,74 nM; $P < 0,05$) is lower than the concentration of NO outside the healthy donors' erythrocytes (2,3±0,75 nM).

4.4 - Level of Intraerythrocytic Nitrite

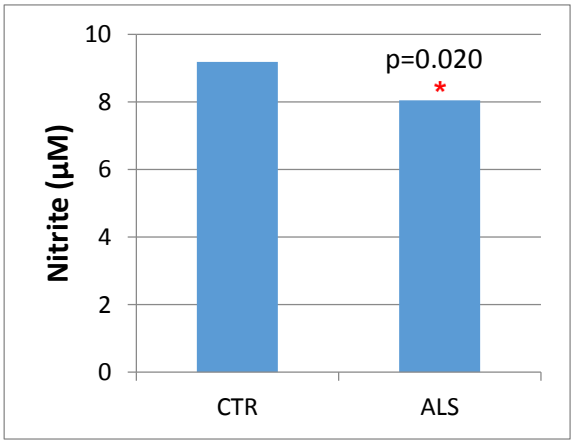

Figure 8 - Level of intraerythrocytic nitrite in the two groups of study.

Figure 8 represents the different concentrations of nitrite (in µM) inside the erythrocytes of both groups.

It is possible to observe a statistically significant difference between the patients and healthy donors. In comparison, people with the disease have lower concentration of nitrite inside their erythrocytes (8,1±2,55 µM; $P < 0,05$), in relation to the control group (9,2±2,36 µM).

4.5 - Level of Intraerythrocytic Nitrate

Figure 9 represents the different concentrations of nitrate (in µM) inside the erythrocytes of both groups.

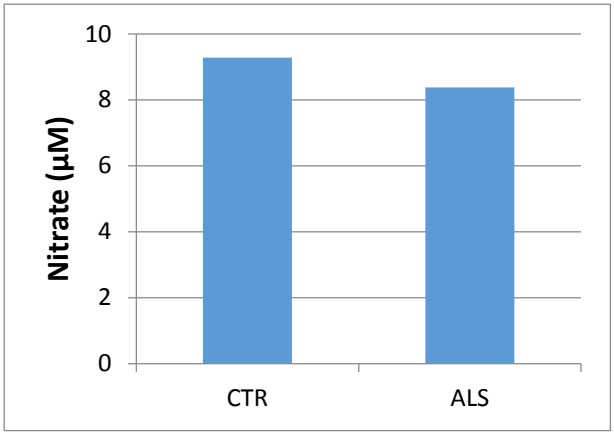

Figure 9 - Level of intraerythrocytic nitrate in the two groups.

As it is possible to examine in the figure, no statistically significant difference is observed in the concentration of nitrate inside the erythrocytes of healthy donors (9,28±2,60 µM) and patients (8,38±2,36 µM).

4.6 - Level of Intraerythrocytic GSNO

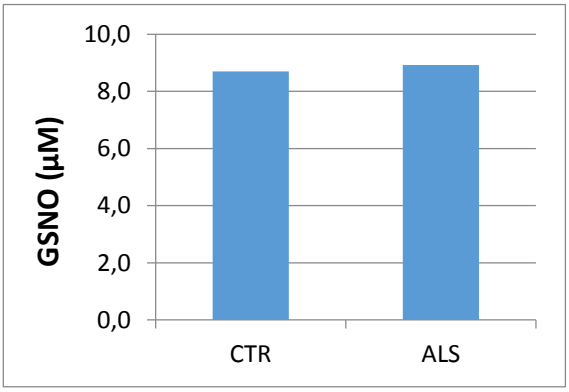

Figure 10 - Concentrations of GSNO inside the erythrocytes of healthy donors and patients with ALS.

The concentration of S-nitrosogluthathione inside the erythrocytes of both groups analysed is illustrated in figure 10.

There is not a significant variation between the control group (8,7±2,5 µM) and patients (8,9±1,8 µM), considering their RBC concentration of GSNO.

4.7 - Enzymatic Activity of AChE

Figure 11 represents the enzymatic activity of AChE (in U/min/mgHb) in erythrocytes of both groups.

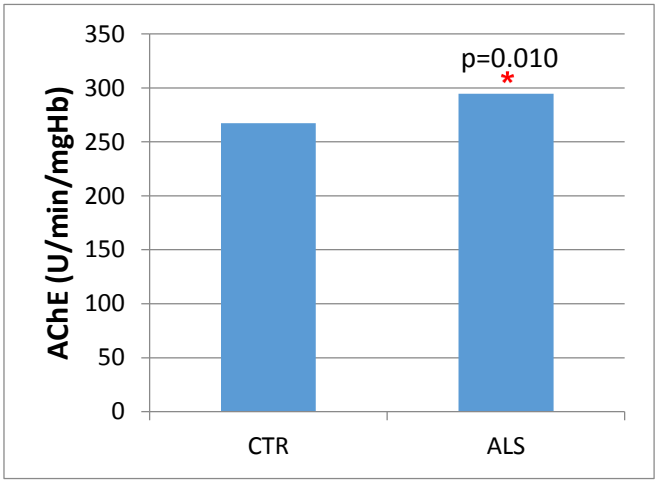

Figure 11 - Comparison of AChE enzymatic activity between the two groups of study.

Observing the graph, it is noteworthy that there is a significant statistically difference between the control group and patients with ALS.

Actually, people with the disease demonstrate a considerably higher AChE activity (295±55 U/min/mgHb; $P < 0,05$) in comparison to the control group (267±45 U/min/mgHb).

4.8 - Profile of Metabolic Parameters of NO Analysed Exclusively in Patients with ALS

In order to estimate if there is an association between the parameters studied, a second analysis of variance was performed, only with the results of patients with ALS. The significant results are illustrated below.

4.8.1 - Profile of Erythrocyte Aggregation by Quartiles of NO in ALS group

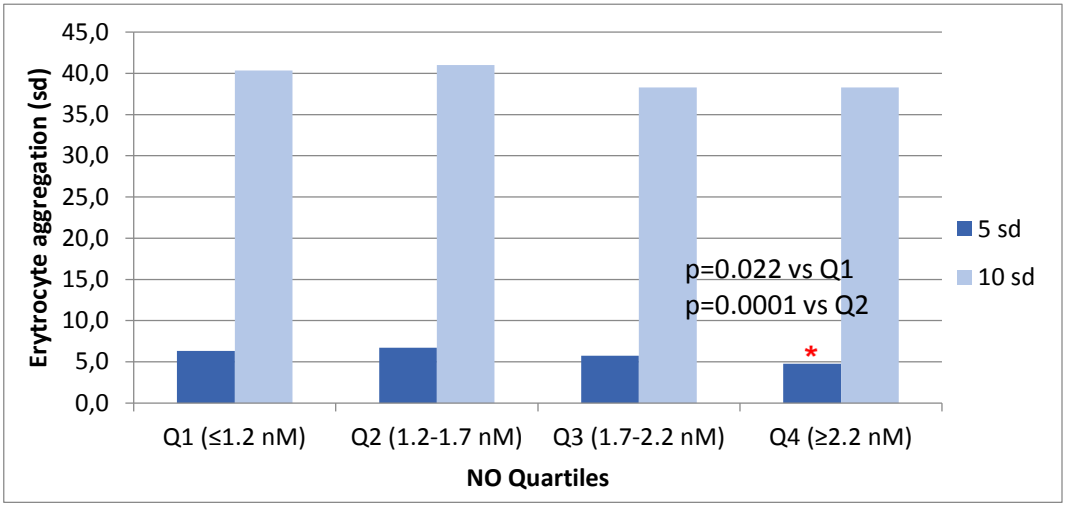

Figure 12 - Relation between erythrocyte aggregation and different range values of NO.

Figure 12 illustrates that Q4 (NO≥2.2 nM; 4,8±2,0 sd; P<0,05) has a statistically significant difference from quartiles Q1 (≤1.2 nM; 6,3±2,1 sd) and Q2 (1.2-1.7 nM; 6,7±1,8 sd), regarding erythrocyte aggregation in period of analysis of 5 seconds.

This demonstrates that for higher concentrations of NO, RBCs of patients with ALS form less aggregates when blood is in stasis for 5 seconds.

4.8.2 - Profile of Erythrocyte Deformability by Quartiles of NO

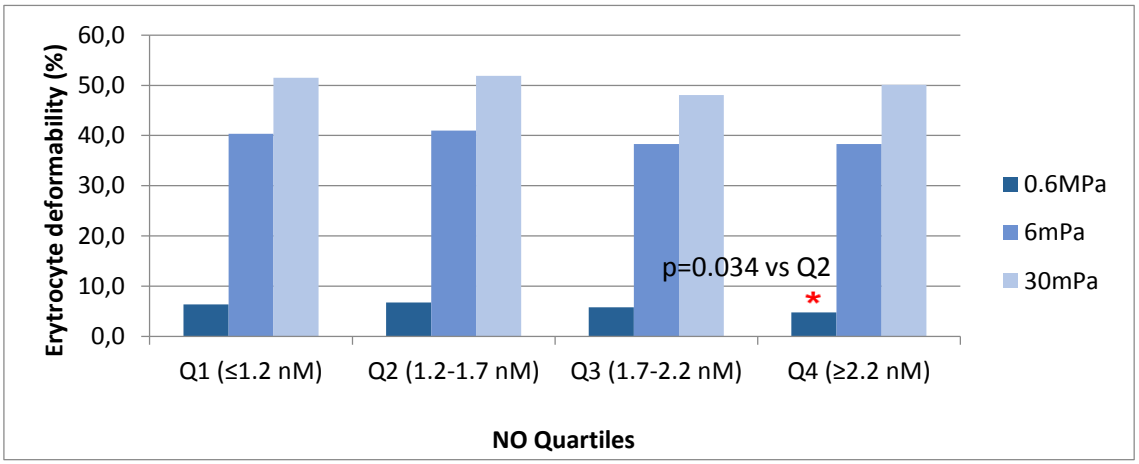

Figure 13 - Values of erythrocyte deformability by quartiles of NO.

The figure shows that Q4 (≥2.2 nM; 4,8±2,0 %; P<0,05) has a statistically significant difference in relation to Q2 (1.2-1.7 nM; 6,7±1,8 %), regarding erythrocytes deformability when subjected to shear forces of 0,6 mPa.

This demonstrates that for higher concentration values of NO, RBCs of patients with ALS have less deformability at lower values of shear stress.

4.8.3 - Profile of Nitrite Values by Quartiles of AChE

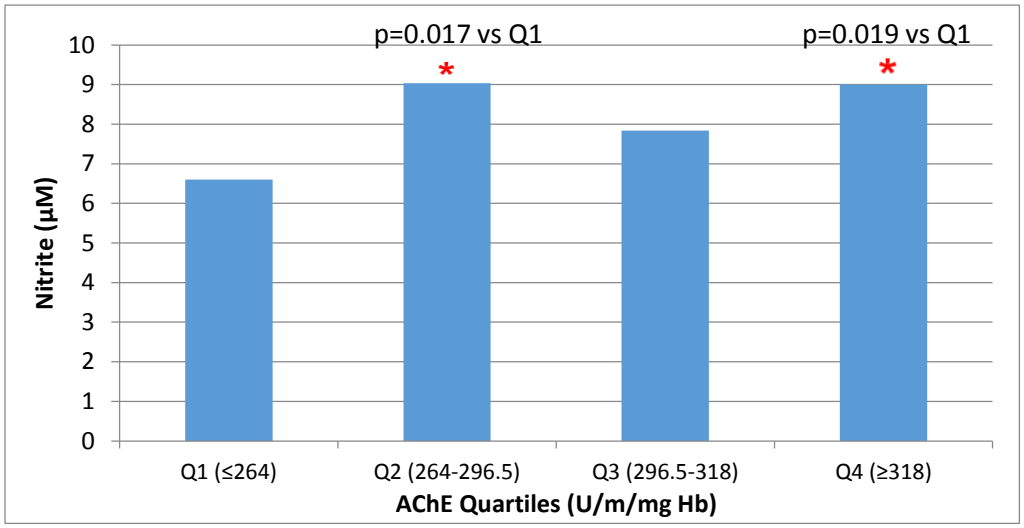

Figure 14 - Profile of nitrite by quartiles of AChE.

The figure shows that Q2 (264-296.5 U/m/mg Hb) and Q4 (≥318 U/m/mg Hb) have a statistically significant difference in relation to Q1 (≤264 U/m/mg Hb), considering the concentration of nitrite (Q1: 6,6±2,25 µM; Q2: 9,0±2,25 µM; Q4: 9,0±2,63 µM; $P < 0,05$).

This clarifies that some range values of enzymatic activity of AChE (Q2 and Q4) are associated with higher concentrations of nitrite inside the RBCs of patients with ALS.

4.8.4 - Profile of Respiratory Function by Quartiles of NO

Another parameter analysed in this project, whenever possible (57 participants), since it is not performed through laboratory analysis and requires the contribution of the patients, was the Respiratory Function (expressed as FVC %).

Figure 15 illustrates that Q4 (≥2.2 nM) has a statistically significant difference from quartiles Q1 (≤1.2 nM) and Q2 (1.2-1.7 nM), regarding the respiratory function of patients with ALS.

This demonstrates that for higher concentrations of NO efflux from the erythrocyte (Q4: 85±27,71 %; P<0,05), the Respiratory Function is lower in comparison to inferior values of NO efflux (Q1: 90±38,39 %; Q2: 95±15,82 %).

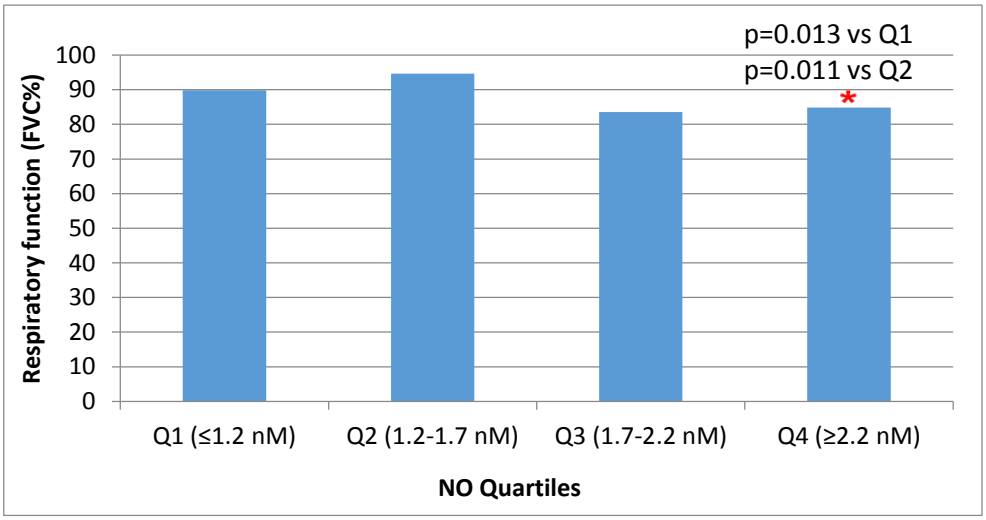

Figure 15 - Association between respiratory function and NO quartiles.

4.9 - Parameters concerning gender

Apart from the analysis that compares the two groups in study (CTR and ALS), gender was also taken into account. However, no statistically significant differences were observed between genders, considering each one of the parameters. The graphs of this investigation can be examined in Annex 2.

5. Discussion

Amyotrophic Lateral Sclerosis is a neurodegenerative disease in which motor neurons die. As a multi-systemic and multi-factorial disorder, it may affect neuronal and non-neuronal cells and it is largely associated with a variety of pathologic events. Knowing that plasma nitrite is one of the major molecules increased in the disease and that erythrocytes function as a reservoir and scavenger of NO, it is of considerable interest to study NO translocation and parameters of nitrogen oxidative stress (such as nitrite and nitrate) in erythrocytes.

This study shows that the values of erythrocyte deformability and membrane enzyme AChE catalytic activity are augmented in the erythrocytes of patients with ALS. This is in agreement with previous studies reporting a signal transduction mechanism hypothesis through which the activation of AChE, present in the membrane of erythrocytes, indirectly leads to the increase of deformability (46).

In more detail, the pathway is initiated with the binding of circulating ACh to membrane AChE. This requires the existence of ACh in human blood, produced by T lymphocytes and endothelial cells, as it happens in inflammatory situations, such as in a disease state, as Kawashima *et al.* and Kirkpatrick *et al.* (46) reported in their studies. Once the complex ACh-AChE is active, protein of membrane band-3 associates with protein Gi, in its subunits Gαi1/Gαi2 and Giβ, what results in conformational changes. As a consequence of these modifications, there is an increase in protein C kinase activity in the RBC membrane, which provokes phosphorylation of membrane proteins, such as protein 4.1R, 4.2 or band-3, and promotes a decrease in the interactions between erythrocyte membrane and cytoskeleton, increasing deformability values (32). Consequently, it is expected a higher NO efflux from the erythrocyte and a higher concentration of NO metabolites inside the cell (69). Nevertheless, in this study, despite the elevated erythrocyte deformability and AChE activity in patients with ALS, the efflux of NO from the RBC and the concentration of NO metabolites inside the RBC are lower in the disease in comparison to the normal physiological situation.

The augmented AChE activity can be justified by the fact that this enzyme is a marker of systemic inflammation (70). Considering this, it would be expected a normal signal transduction pathway, with the efflux of NO from the erythrocytes. Since this does not happen in this study, it is plausible to hypothesise a blockage of the mechanism, probably at the level of kinases, responsible for the phosphorylation of the membrane proteins. This would justify the maintenance of NO inside the erythrocyte, but not the elevated deformability, which would possibly be augmented by a plasmatic factor that influences this hemorheologic property but not the erythrocyte aggregation.

On the other hand, it is also possible to assume that the population of erythrocytes of ALS may present a higher percentage of AChE in relation to healthy donors or a higher rate of RBCs turnover in the disease, although this is not described yet. In these cases, the enzyme could be augmented without changing the conformation of erythrocyte membrane, whereby there may be an activation of protein Gi without the remainder signal transduction pathway. Thus, the mechanism would not occur, as well as the outlet of NO from the erythrocyte. Once again, the elevated deformability could be then justified by the influence of a plasmatic factor (or not if the phosphorylation of cytoskeleton proteins occur).

Nevertheless, other supposition is reasonable. Since AChE and deformability are elevated it is expected that the pathway is, at least at some extent, working normally. Therefore, the maintenance of NO inside the RBCs could be justified by the influence of some plasmatic factor that blocks its output. In previous studies of Saldanha *et al.* it was postulated that fibrinogen, when in presence of ACh, binds to CD47 of the erythrocyte membrane, increasing the erythrocyte deformability and preventing the efflux of NO from the RBC (71). In fact, ALS is associated with inflammation and fibrinogen was found to be elevated in these patients (72) in comparison to controls. Furthermore, fibrinogen was shown to have an important role in the progression of neurodegenerative diseases (73). Accordingly to this, if high levels of fibrinogen are present in this disorder, NO delivery by erythrocytes might be compromised by their NO scavenging ability. Consequently, this may act as a compensatory mechanism against the overproduced NO by endothelial inducible nitric oxide synthase (71) and can represent a benefit to the intravascular resolution of inflammation (74). However, if this hypothesis is accurate, it was expected to be an increase of nitrite and nitrate inside the erythrocyte (71), once fibrinogen maintain NO there. Nevertheless, the opposite was observed, since intraerythrocytic nitrite were diminished in patients with ALS. This is in agreement with the increase of nitrate in serum of ALS patients, as suggested by Taskiran *et al.* (75).

Other possible factor capable of influence the efflux of NO from the erythrocyte is MetHb. It is known that high concentrations of NO oxidize and denature Hb, forming both metHb and Heinz bodies, both of which are associated with the membrane and cytoskeletal proteins. Huang *et al*. demonstrated that when MetHb binds to the membrane of the RBC, no efflux of NO is observed (43).

Nonetheless, neither fibrinogen nor MetHb were measured in this study.

NO has an essential role at regulating neural functions, as referred in the section 1.1.2) Pathogenesis. A study concerning about its impact on neuronal survival during oxidative stress, showed that NO functions as an endogenous physiological modulator of energy conservation for the brain, since it inhibits cytochrome c oxidase activity in neurons and glia, resulting in down-regulation of mitochondrial energy production. This neuronal energy metabolism is controlled by tuning the balance of glucose-6-phosphate consumption between glycolysis and pentose–phosphate pathway (76).

Nevertheless, this molecule also acts as a modulator of glycolysis in the RBCs, by promoting the translocation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD) from the membrane to the cytosol. Some NO donors, such as peroxynitrite, or thiols are also known to increase GAPD activity, glycolysis, measured as lactate production, and energy charge levels (77).

In conclusion, it is plausible to assume that in a disease such as ALS, characterized by oxidative stress, NO exists in higher amounts inside the RBCs as a participant in the metabolism of erythrocytes and a trigger factor of pathogenesis. Nonetheless, this hypothesis still needs to be confirmed.

Regarding the quantification of NO metabolites, it was verified that nitrite, with particular statistical significance, were reduced inside the erythrocytes of patients with ALS in comparison to healthy donors. This is not in agreement with the low efflux of NO from the RBC, since in a normal situation, NO (in this case, inside the erythrocyte) would conjugate with superoxide anion, resultant from the auto-oxidation of intraerythrocytic Hb, and produce peroxynitrite that decompose in nitrite and nitrate. Moreover, as presented in Figure 4, these metabolites can be also produced from oxyhaemoglobin and glutathione, resulting compounds from the signal transduction pathway. Therefore, the malfunction of this mechanism may explain the low concentration of nitrite and nitrate inside the erythrocytes.

However, we must not discharge a higher efflux of both derivatives from the RBCs, contributing to their increased levels in blood circulation. In fact, NO is also produced by endothelial cells, outside the erythrocytes, which enhances the level of plasmatic nitrite. Actually, plasma nitrite have been described as an index of eNOS activity in the circulation in humans. Thereby, the reduced levels of nitrite inside the RBC could be taken as a compensatory mechanism in disease states, through which the erythrocyte does not increase its oxidative stress. This is confirmed by the fact that intraerythrocytic nitrite reacts with oxyhemoglobin to produce methemoglobin and nitrate (78), another metabolite possibly involved in oxidative stress.

It was of extreme interest to establish an association between the different parameters possibly involved in this disease. With that purpose, an analysis of quartiles was made, in which AChE and NO were divided into four ranges of values and crossed with all the parameters studied.

Although erythrocyte deformability and aggregation showed a statistically significant association with NO efflux values, the differences are so light that it is not possible to draw conclusions.

None of the other parameters demonstrated an association between each other.

First of all, it is important to note that although the AChE activity is generally augmented in the disease, different patients present variable values that may go from normal values to higher ones.

Furthermore, even though nitrite are at low levels inside the erythrocytes of all patients with ALS, there are two range values of AChE enzymatic activity (normal values: 264-296.5 U/m/mg Hb and elevated values: ≥318 U/m/mg Hb) at which nitrite are increased.

Through the association evidenced in *in vitro* studies concerning AChE activity and NO efflux, it is possible to verify that the range of values of AChE activity where NO is increased correspond to the same range of values where nitrite are also elevated. This makes it reasonable to assume that there is higher efflux of NO for values of AChE activity in which nitrite are increased inside the RBC. This could be presumed as a possible compensatory mechanism in which there is more outlet of NO when nitrite are augmented inside the cell and, on the contrary, NO is maintained on the erythrocyte when nitrite exist in lower concentrations.

The respiratory function of patients with ALS was evaluated by measuring their Forced Vital Capacity (FVC). FVC is the volume of air that can forcibly be blown out after full inspiration. Normal values range from 80-120%. A higher percentage of FVC corresponds to a better respiratory function.

In this study, it was observed that for higher concentrations of NO outside the erythrocyte, the respiratory function is worse. Thus, it is possible to assume that when NO is maintained inside the RBC, the respiratory function is easily assured. This is in agreement with Pawloski *et al.*, who defended that erythrocyte acts as a NO scavenger, allowing an important NO mediated blood flow regulation, facilitated by variable oxygen levels. This principle should result in capture of NO in highly oxygenated tissues and release in relatively hypoxic tissues (23), as it may happens in ALS where respiratory muscle weakness causes ventilatory insufficiency and tissue hypoxia (79). Thus, once inside the erythrocyte, considering systemic circulation, NO interacts with Hb and becomes able to regulate the uptake and delivery of oxygen, what justifies its important role inside the RBC, where its destiny is modulated by tissue oxygen partial pressure (PaO2) (80).

Nevertheless, this was not observed in the study, since worse respiratory function was related to a higher NO efflux in patients. Accordingly, other perspective may be taken into account considering the pulmonary system. In fact, the erythrocyte may release more NO when the respiratory function is compromised, so that it can be availed by pulmonary tissues.

6. Conclusion and Final Considerations

The present work provided a more detailed study of erythrocyte as a biological sensor, capable of modulate NO bioavailability, and suggests AChE as a promising biomarker in ALS. Thereby, more studies, including pro and anti-inflammatory mediators, will be of crucial interest. In addition, it was observed that the respiratory function of patients with ALS was worse for higher concentrations of NO outside the erythrocyte, which may give rise to a possible therapeutic strategy in which NO should be preserved inside the RBC.

At present three critical questions are still open: understanding the etiology of the disease, identification of molecular biomarkers and finding effective therapeutic agents.

In fact, although extensive research for the identification of molecular markers in the cerebrospinal fluid and plasma of ALS patients has been made, there are no validated biomarkers for the disease yet (1).

Furthermore, there is no cure for ALS. Nevertheless, Riluzole, was the only medication to receive Food and Drug Administration approval and demonstrate a positive, but modest, effect in the progression of the disease, due to its antiglutamatergic activity, increasing the survival or the time to tracheotomy (1; 5; 81).

Further possible therapeutic strategies have been proposed, based on the pathogenesis of ALS, such as injection of viral vectors that produce RNAi-mediated silencing of mutant SOD1 (1), NOS donor compounds (when disease is associated with deficient S-nitrosylation) (2), and specially targeting of NO or other neurotoxic mediators (like peroxynitrite) with antioxidant therapy without modifying the activity of NOS (16; 35). Actually, assuming that cellular oxidative stress is the principal pathologic mechanism to trigger the disease, novel appropriate treatments can be developed, with less time and economical effort, to slow or even stop progression of this devastating neurodegenerative disease.

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Annexes

Annex 1 – Excerpt of the table that comprises all the results of this study.

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									Deformability			Aggregation		
Participant	Gender	Age	FVC (%)	Hb (g/dL)	NO (nM)	GSNO (µM)	Nitrite (µM)	Nitrate (µM)	0,6 (Pa)	6,0 (Pa)	30,0 (Pa)	5s	10s	AChE (U/min/mgHb)
ALS 1	2	54	120,70	13	1,6	9,24	6,1	7,1	9,04	43,49	54,26	9,6	20,5	332
ALS 2	1	60	120,10	11,4	1,2	11,4	5,6	7,1	8,59	45,53	54	16,2	24,3	239
ALS 3	2	60	96,90	14,3	1,1	10,3	8,6	9,1	6,81	45,56	56,49	7,7	16,6	301
ALS 4	1	57	66,40	12,5	1,1	9,24	8,6	9,6	3,76	35,13	50,76	11,5	17,5	244
ALS 5	1	71	77,40	11,7	1,2	8,16	6,6	7,6	7,89	40	46,18	10,7	19,8	233
ALS 6	2	64	95,90	14	1,5	8,16	6,1	6,6	6,86	40,88	50,16	9,4	17,3	240
CTR1	2	>40	-	13,8	3,2	8,2	10,6	9,6	1,92	33,73	43,79	10,8	25,6	209
CTR2	2	>40	-	13,4	1,7	8,2	12,1	13,6	3,35	36,78	52,17	10,8	15,3	246
CTR3	2	>40	-	15,8	2,1	8,2	13,6	10,6	4,87	33,24	38,03	10,8	31,5	263
CTR4	2	>40	-	18,1	4,2	7,1	9,6	11,6	3,8	34,6	42,04	8,6	14,3	239
CTR5	2	>40	-	16,4	2,2	10,3	9,1	8,1	2,04	31,86	40,16	7	14,2	272
CTR6	2	>40	-	16,2	3,5	8,2	10,1	8,1	5,91	43,3	50,26	6,4	12,8	273

Table 1 - Excerpt of the table that comprises all the results of this study.

Gender: 1 – Woman
2 – Man

FVC (%) - Forced Vital Capacity

Annex 2 - Analysis of parameters in both groups in study (CTR and ALS) concerning gender.

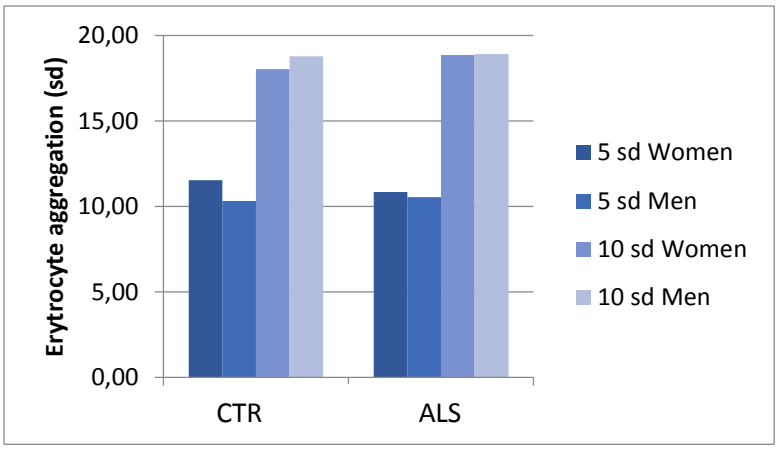

Figure 16 - Comparison of erythrocyte aggregation between controls and patients with ALS concerning gender.

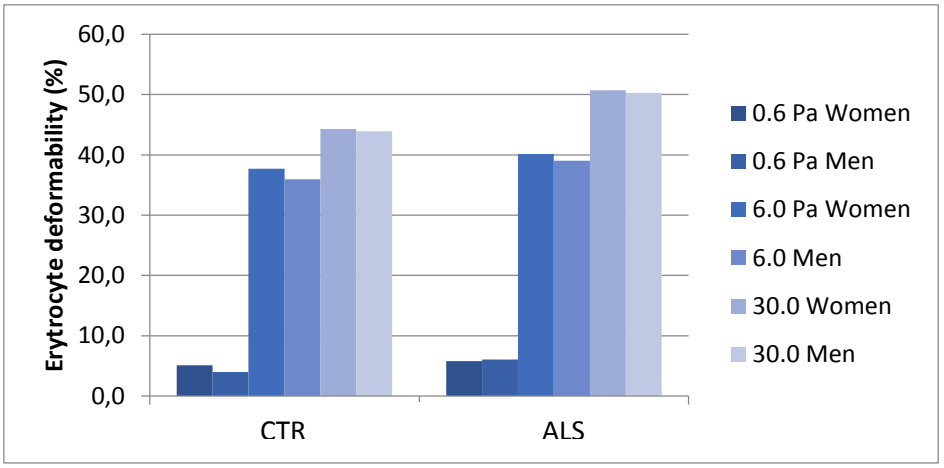

Figure 17 - Comparison of erythrocyte deformability between the two groups concerning gender.

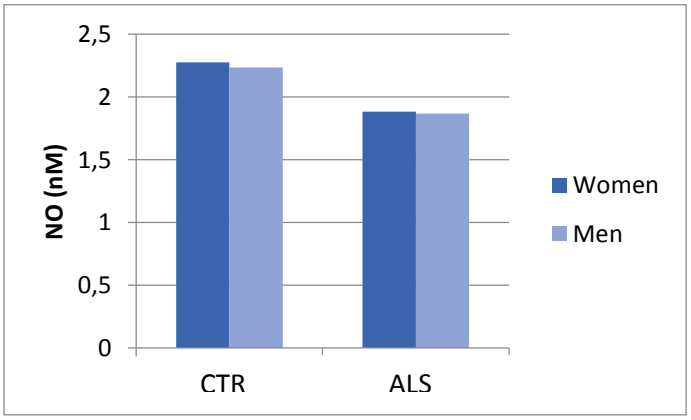

Figure 18 - Comparison of NO efflux between the control and disease group concerning gender.

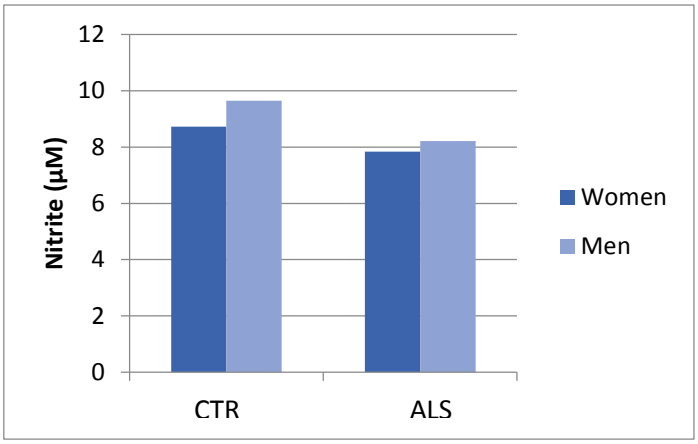

Figure 19 - Level of intraerythrocytic nitrite in the two groups of study concerning gender.

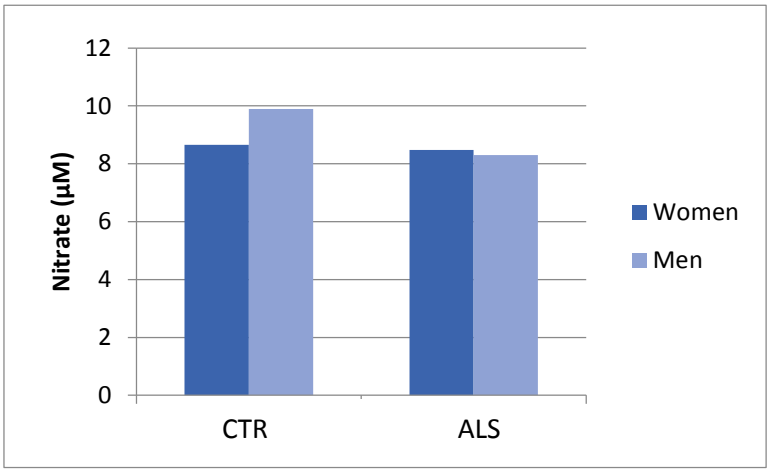

Figure 20 - Level of intraerythrocytic nitrate in the two groups concerning gender.

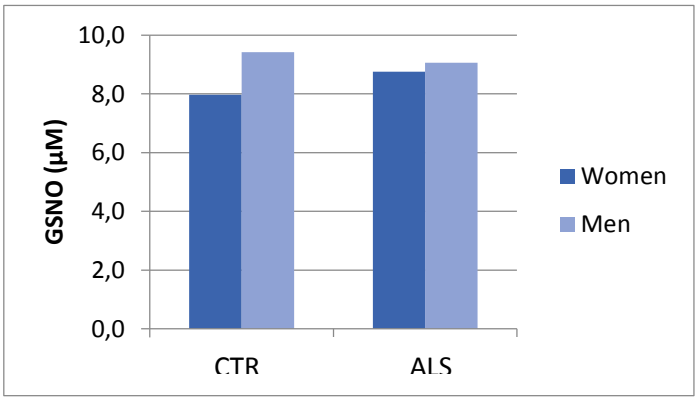

Figure 21 - Concentrations of GSNO inside the erythrocytes of healthy donors and patients with ALS concerning gender.

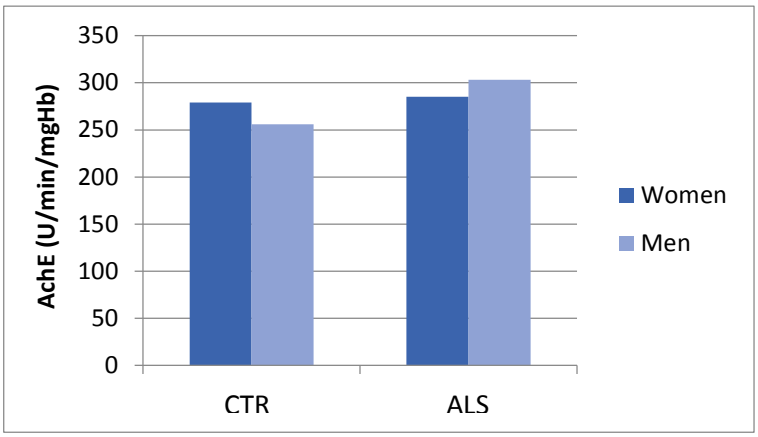

Figure 22 - Comparison of AChE enzymatic activity between the two groups of study concerning gender.